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Isolation and Characterization of a Slowly Milk-Coagulating Variant of *Lactobacillus helveticus* Deficient in Purine Biosynthesis

ELVIRA M. HEBERT,[1] GRACIELA S. DE GIORI,[1,2]* AND RAUL R. RAYA[1]

Centro de Referencia para Lactobacilos (CERELA), CONICET,[1] and Cátedra de Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán,[2] San Miguel de Tucumán, Argentina

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A slowly milk-coagulating variant (Fmc$^-$) of *Lactobacillus helveticus* CRL 1062, designated S1, was isolated and characterized. Strain S1 possessed all the known essential components required to utilize casein as a nitrogen source, which include functional proteinase and peptidase activities as well as functional amino acid, di- and tripeptide, and oligopeptide transport systems. The amino acid requirements of strain S1 were similar to those of the parental strain. However, on a purine-free, chemically defined medium, the growth rate of the Fmc$^-$ strain was threefold lower than that of the wild-type strain. *L. helveticus* S1 was found to be defective in IMP dehydrogenase activity and therefore was deficient in the ability to synthesize XMP and GMP. This conclusion was further supported by the observation that the addition of guanine or xanthine to milk, a substrate poor in purine compounds, restored the Fmc$^+$ phenotype of *L. helveticus* S1.

Lactobacillus helveticus is used in the dairy industry with other lactic acid bacteria (LAB) as starter cultures to produce fermented milk, sour milk, and Swiss- and Italian-type cheeses. A useful guide to the suitability of these bacteria for cheese-making is their ability to coagulate autoclaved milk within 16 h at 42°C when used as a 1% freshly coagulated inoculum (35), a property which defines a fast milk-coagulating (Fmc$^+$) strain (an Fmc$^-$ strain requires more than 30 h of incubation for coagulating autoclaved milk at 42°C). Thus, the ideal starter culture should rapidly and dependably produce lactic acid during growth in milk. For this, LAB depend on their ability to metabolize lactose and on the presence of a complete proteolytic system which allows the efficient degradation and utilization of casein, the major milk protein. The specialized proteolytic system of these microorganisms consists of a cell envelope-associated proteinase, transport systems to allow uptake of the resultant amino acids and peptides, and several intracellular peptidases which degrade peptides to amino acids (17).

In *Lactococcus*, the conversion of an Fmc$^+$ phenotype to an Fmc$^-$ phenotype has mainly been attributed to loss of either a cell wall-bound proteinase (PrtP) or the enzyme(s) required for lactose utilization and can be due to loss of a plasmid coding for these enzymes (16, 20). Yu et al. (36) found that the Fmc$^-$ phenotype in *Lactococcus lactis* could also be due to loss of a plasmid encoding an oligopeptide permease system. In addition, it was reported that an aminopeptidase (encoded by *pepA*) is required for optimal growth of *L. lactis* in milk (19). Recent findings have shown that *Lactococcus lactis* subsp. *lactis* C2 deficient in aspartate synthesis exhibited an Fmc$^-$ phenotype (35). When any of these properties are lost from the cells, the ability to synthesize lactic acid is also slowed and the cells are no longer effective starter organisms. Despite the industrial importance of thermophilic lactobacilli (e.g., *L. helveticus*) as dairy starters, information about the nature of the Fmc$^-$ phenotype in these microorganisms is limited.

L. helveticus strains show phenotypic and genotypic variability (7, 8, 10, 11). Fortina et al. (7) reported that a wide phenotypic variability exists among *L. helveticus* strains isolated from natural cheese starters. Cell heterogeneity affected different phenotypes such as resistance to lysozyme (7, 34), sugar fermentation, phage resistance, and proteolytic activity (7, 29). Morelli et al. (23) and Reinheimer et al. (29) reported the presence of spontaneous Fmc$^-$ isolates within the *L. helveticus* strains HLM 1 and ATCC 15807, respectively, and have suggested the possibility of a linkage between casein hydrolysis and the Fmc$^-$ phenotype. In previous studies, we have demonstrated that *L. helveticus* CRL 1062, a starter used for the manufacture of Argentinian hard cheeses, exhibits an Fmc$^+$ phenotype (13). *L. helveticus* CRL 1062 is auxotrophic for aspartate (14); therefore, this strain may be able to utilize some of the casein-derived aspartate-containing oligopeptides. In this work, we describe the isolation and characterization of strain S1, a slow-milk-coagulation variant of *L. helveticus* CRL 1062. We show that, in addition to a functional proteolytic system, *L. helveticus* CRL 1062 requires an IMP dehydrogenase activity to exhibit an Fmc$^+$ phenotype. This enzyme is necessary to provide sufficient GMP to allow the organism to grow to high cell densities in milk.

MATERIALS AND METHODS

Microorganisms, media, and growth conditions. *L. helveticus* CRL 1062 was obtained from CERELA (Centro de Referencia para Lactobacilos, Argentine). Cultures were stored at −70°C in 10% sterile reconstituted skim milk (RSM) containing 0.5% yeast extract and 10% glycerol and were reactivated in MRS (1) broth at 42°C for 16 h. Slowly milk-coagulating variants of *L. helveticus* CRL 1062 were isolated on casein medium (CM). CM is a modification of the casein-based medium described by Morelli et al. (23), where the concentrations of tryptic digest of casein, yeast extract, and $MnSO_4 \cdot H_2O$ were reduced five times. CM contains the following (in grams per liter): tryptic digest of casein, 1; yeast extract, 0.4; Tween 80, 1; cysteine, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.04; sodium caseinate, 1; trisodium citrate, 4.4; glucose, 10; and agar, 12.

The composition of the simplified chemically defined medium (SCDM) has been described previously (14). This medium contained the following (in grams

* Corresponding author. Mailing address: CERELA, Chacabuco 145, 4000 San Miguel de Tucumán, Argentina. Phone and fax: 54-381-4310465. E-mail: gsayoy@cerela.org.ar.

per liter): glucose, 10; KH_2PO_4, 3; K_2HPO_4, 3; sodium acetate, 5; $MgSO_4 \cdot 7H_2O$, 0.2; L-alanine, 0.1; L-arginine, 0.1; L-asparagine, 0.2; L-aspartic acid, 0.2; L-cysteine, 0.2; L-glutamine, 0.2; L-glutamic acid, 0.2; glycine, 0.1; L-histidine, 0.1; L-isoleucine, 0.1; L-leucine, 0.1; L-lysine, 0.1; L-methionine, 0.1; L-phenylalanine, 0.1; L-proline, 0.1; L-serine, 0.1; L-threonine, 0.1; L-tryptophan, 0.1; L-tyrosine, 0.1; L-valine, 0.1; uracil, 0.01; nicotinic acid, 0.001; calcium pantothenate, 0.001; pyridoxal, 0.002; and riboflavin, 0.001. It also contained Tween 80 at 1 ml/liter. When indicated, SCDM was supplemented with the following (in milligrams per liter): guanine, 10; adenine, 10; inosine, 5; orotic acid, 5; folic acid, 1; vitamin B_{12}, 1; thiamine, 1; biotin, 10; and *p*-aminobenzoic acid, 10. This supplemented SCDM was designated CDM.

All amino acids, vitamins, bases, and inorganic salts were of analytical grade (Sigma Chemical Co., St. Louis, Mo.). Defined media were adjusted to pH 6.5 and sterilized by passing them through a 0.2-μm-pore size sterile filter (Gelman Sciences, Ann Arbor, Mich.). In some experiments, SCDM was supplemented with 1% sodium caseinate (wt/vol; Sigma) and 1.5% agar (wt/vol).

Growth experiments with *L. helveticus* on SCDM and CDM were conducted as follows. Bacterial cells, propagated in MRS at 42°C for 16 h, were harvested by centrifugation at 10,000 × *g* for 15 min, washed twice in sterile 50 mM sodium phosphate buffer (pH 7.0) to eliminate carryover nutrients, and resuspended in the same buffer to the original volume. This cell suspension was used to inoculate the different media at an initial optical density at 560 nm (OD_{560}) of 0.07. Bacterial growth was then monitored at 42°C by measuring the OD_{560}.

Coagulation tests. Washed cells, prepared as described above, were used to inoculate (1%) RSM, RSM supplemented with 1% glucose (RSM-G), and RSM supplemented with 0.25% yeast extract (RSM-YE). Cells were then incubated at 42°C for 16 h.

Cell suspensions and CE. Cells grown in the different media were harvested by centrifugation (10,000 × *g*, 15 min, 4°C) at the mid-exponential growth phase (OD_{560} = 0.65), washed twice with 0.85% (wt/vol) NaCl supplemented with 10 mM $CaCl_2$, and resuspended to a final OD_{560} of approximately 10 in 100 mM sodium phosphate buffer (pH 7.0). The washed whole cells were allowed to utilize the residual sugar and intracellular amino acids for 30 min at 42°C. Cell extracts (CE) were obtained by vortexing the bacterial cell suspensions with glass beads (0.15- to 0.25-mm diameter; Sigma) at a ratio of 1:1 and then kept on ice for 1 min each. This step was repeated seven times. Glass beads, cell debris, and unbroken cells were removed by centrifugation (10,000 × *g*, 10 min, 4°C).

Enzyme assays. Proteinase (PrtH) activity of whole-cell suspensions was measured in 50 mM phosphate buffer, pH 7.0, at 42°C with the chromogenic substrate succinyl-alanyl-alanyl-prolyl-phenylalanine-*p*-nitroaniline (S-Ala; Sigma) as described by Exterkate (5). PrtH activity was measured from the initial part of the progress curve, where release of the *p*-nitroaniline was linear with time. One unit of proteinase was defined as the amount required to liberate 1 nmol of nitroanilide per min. Specific activity was expressed as proteinase units per milligram of protein. Cell lysis was determined by following the release of lactate dehydrogenase by the method of Thomas (33).

β-Galactosidase activity was determined in cells grown in MRS broth with 1% (wt/vol) lactose instead of glucose or RSM according to the method of Miller (21). The release of *o*-nitrophenol (ONP) from the substrate *o*-nitrophenyl-β-D-galactopyranoside (Sigma) was determined by spectrophotometric measurements at 420 nm. Specific activity was expressed as micromoles of ONP released per milligram of protein per minute.

IMP dehydrogenase activity was assayed by measuring the increase in absorbance at 290 nm owing to the formation of XMP from IMP at 37°C (9). The reaction mixture contained the following in a total volume of 1 ml: 50 mM KCl, 5 mM reduced glutathione, 1.25 mM NAD^+, 50 mM Tris-HCl buffer (pH 8.0), and 1.5 mM IMP. The reaction was initiated by the addition of CE to the complete system. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmol of XMP per min at 37°C. Specific activity is expressed as micromoles of XMP produced per minute per milligram of protein. The XMP produced was calculated from a molar coefficient of 4,600 liters/mol · cm at 290 nm and pH 8.0 (9).

GMP synthetase activity was assayed as previously described (27). The reaction mixture contained 70 mM HEPES buffer (pH 8.2), 10 mM $MgCl_2$, 20 mM glutamine, and 0.3 mM XMP in a total volume of 1 ml. The reaction was initiated by the addition of CE to the complete system. After 20 min at 37°C, the reaction was stopped by placing the reaction mixture in a boiling water bath for 2 min. The reaction mixture was cooled to 37°C, and the amount of glutamate produced was determined with the test combination for glutamic acid (Boehringer, Mannheim, Germany). One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmol of glutamate per min at 37°C. Specific activity is expressed as micromoles of glutamate produced per minute per milligram of protein.

Casein hydrolysis. Washed cells, harvested from CDM, were suspended in 100 mM sodium phosphate buffer (pH 7.0) and allowed to utilize the residual intracellular amino acids for 30 min at 42°C. Casein degradation was conducted as described previously (12). Washed whole cells (OD_{560} = 10) were incubated with 5 mg of substrate/ml dissolved in 100 mM phosphate buffer (pH 7.0) at a ratio of 1:1 (vol/vol). α- or β-casein (Sigma) was used as the substrate. The resulting mixtures were incubated at 42°C for 3 h, the samples were centrifuged, and the supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (18). Either Coomassie brilliant blue R-250 or silver staining (Bio-Rad Laboratories, Hercules, Calif.) was used to visualize the proteins after SDS-PAGE.

Protein determination. Protein concentration was determined by using a protein assay according to the manufacturer's instructions (Bio-Rad).

DNA isolation, amplification, and sequencing. Genomic DNA of *L. helveticus* was isolated as described previously (28). The primers (A2, 5′-GTTATCTCTG CTGGGAACTC-3′, and A4, 5′-GAAAAAGCCCATGTATGG-3′) were designed from an alignment of the conserved regions of the proteinase genes from different LAB, and they were used to amplify the region surrounding the active-site residues of the proteinase gene of *L. helveticus*. All primers were synthesized by Gibco-BRL Custom Primers (Grand Island, N.Y.). The PCR assay was performed with 50 μl containing 30 ng of bacterial DNA, 2.5 mM $MgCl_2$, 100 μM concentrations of each of four deoxynucleoside triphosphates, 1 μM concentrations of each primer in *Taq* buffer (Gibco BRL), and 2.5 U of *Taq* polymerase (Gibco BRL). PCR was carried out in a programmable heating incubator (Perkin-Elmer Corp., Norwalk, Conn.) for 30 cycles. The cycling program used was 30 cycles of 94°C (1 min), 50°C (1 min), and 72°C (1.5 min). Amplification products were analyzed by electrophoresis in 1% agarose gels containing 200 μg of ethidium bromide (Sigma)/liter. PCR products were purified with a Prep-A-Gene master kit (Bio-Rad), and DNA sequencing was performed by the Bior-Resource Center (Ithaca, N.Y.).

RESULTS

Isolation of an Fmc$^-$ derivative from *L. helveticus* CRL 1062. Cell heterogeneity within *L. helveticus* strains HLM 1 (23) and ATCC 15807 (29) has been described. With *L. helveticus* CRL 1062, we initially failed to isolate Fmc$^-$ variants using the casein-based medium described by Morelli et al. (23). In this medium, regardless of the size of single colonies of *L. helveticus* CRL 1062, all tested isolates (from large and small colonies) coagulated RSM in 16 h at 42°C. This could be due to the fact that this isolation medium contains yeast extract (2%) and tryptic digest of casein (5%) in addition to sodium caseinate (1%). In addition to serving as a nitrogen source, the first two compounds might also provide essential nutrients for optimal growth of the Fmc$^-$ variants of CRL 1062 strain likely present in the culture. However, in the new CM, where the concentration of tryptic digest of casein and yeast extract was reduced fivefold, large (>2.5 mm) colonies, surrounded by a white precipitation halo, and pinpoint colonies could be ascribed to the Fmc$^+$ and Fmc$^-$ phenotypes, respectively. Fmc$^-$ variants were observed with a frequency of 1%. An Fmc$^-$ derivative, designated S1, was isolated and further characterized.

Growth characteristics and β-galactosidase and proteinase activities of S1. *L. helveticus* S1 required up to 30 h to coagulate RSM milk at 42°C; therefore, this strain exhibited an Fmc$^-$ phenotype. As the ability of LAB to coagulate RSM depends mainly on their capacity to metabolize lactose and to hydrolyze casein, it was necessary to determine which, if any, of these components were missing in *L. helveticus* S1. Addition of yeast extract, but not glucose, to RSM restored the Fmc$^+$ phenotype of S1 (Table 1). Wild-type strain CRL 1062 and its derivative S1 displayed similar β-galactosidase activities (Table 1). Furthermore, these strains had similar levels of proteinase

TABLE 1. Growth characteristics and β-galactosidase and proteinase activities of *L. helveticus* CRL 1062 and its slowly milk-coagulating variant S1

Strain	Growth[a]			Sp act (mean ± SD)	
	RSM	RSM-G	RSM-YE	β-Galactosidase[b]	Proteinase[c]
CRL 1062	+	+	+	6.6 ± 0.2	6.1 ± 0.2
S1	−	−	+	6.5 ± 0.3	6.2 ± 0.2

[a] Strains were inoculated (2%) into the indicated media and incubated for 16 h at 42°C. Growth was monitored as milk coagulation (+).
[b] Micromoles of ONP liberated per microgram of protein per minute at 42°C.
[c] Nanomoles minute^{-1} milligram of protein^{-1}. The lactate dehydrogenase activity was less than 0.06% of the total activity in each cell extract.

TABLE 2. Maximal specific growth rate of *L. helveticus* CRL 1062 and S1 in SCDM supplemented with different groups of nucleotides and vitamins

Strain	μ_{max} (h^{-1}) in[a]:							
	CDM	SCDM	SCDM supplemented with group[b]:					
			A	B	C	D	E	F
CRL 1062	0.36	0.34	0.35	0.36	0.34	0.36	0.35	0.35
S1	0.35	0.10	0.09	0.36	0.09	0.09	0.10	0.35

[a] Values are averages from at least three independent experiments. Standard deviations were <5%.
[b] Different pools of vitamins and nucleotides were used to characterize the specific requirements of *L. helveticus* S1. Group A, folic acid, vitamin B_{12}, and thiamine; group B, adenine, inosine, and guanine; group C, orotic acid, biotin, and *p*-aminobenzoic acid; group D, folic acid, inosine, and orotic acid; group E, vitamin B_{12}, adenine, and biotin; group F, thiamine, guanine, and *p*-aminobenzoic acid.

activity (evaluated on the chromogenic substrate S-Ala) (Table 1) and exhibited identical hydrolytic patterns with α- and β-casein as substrates (Fig. 1). PCR analysis also confirmed that S1 carried the proteinase genes of *L. helveticus* (data not shown). These data indicate that in S1, lactose metabolism and proteinase activity are functional.

Peptide transport systems of *L. helveticus* S1. *L. helveticus* strains are auxotrophic for leucine (14, 24). To establish the role of di-, tri-, and oligopeptide transport systems in the utilization of casein-derived peptides, cell growth of *L. helveticus* CRL 1062 and its variant S1 was evaluated in leucine-free CDM supplemented with several leucine-containing peptides and compared to their growth in CDM. No differences in growth rate were observed between CRL 1062 and S1 in CDM (Table 2) and in leucine-deficient CDM containing either the dipeptide Leu-Pro, the tripeptide Leu-Gly-Gly, or the oligopeptide Leu-Leu-Val-Tyr-Ser. These results indicated the functionality of the amino acid and di-, tri-, and oligopeptide transport systems as well as the functionality of the intracellular peptidases which were able to utilize these peptides as a leucine source.

Nutritional requirements of *L. helveticus* S1. CRL 1062 and S1 (Fmc^+ and Fmc^-, respectively) exhibited similar growth rates in CDM. However, the growth rate of S1 in SCDM was threefold lower than that of the parental strain (Table 2). CDM is an SCDM-based medium supplemented with a pool of bases (guanine, adenine, uracil, inosine, and orotic acid) and vitamins (folic acid, vitamin B_{12}, thiamine, biotin, and *p*-aminobenzoic acid). To characterize the specific base and vitamin requirements of S1 with respect to the wild-type strain, the nucleotides and vitamins were divided into six groups (Table 2). The strains were then grown on SCDM supplemented with each of the six groups of vitamins and nucleotides. No changes in the growth rate were observed when the variant S1 was cultured in SCDM supplemented with group A, C, D, or E (Table 2). However, the supplementation of SCDM with group B or F stimulated the growth of S1, and this growth rate was comparable to that obtained on CDM (Table 2). These results suggested that synthesis of guanine was affected in *L. helveticus* S1, since this base was present in groups B and F. This was confirmed when the growth rate of S1 was determined in SCDM supplemented with guanine, inosine, or adenine (Fig. 2). S1 grew at the same rate as the parental strain on SCDM containing guanine, but not on SCDM supplemented with adenine or inosine. Furthermore, the S1 growth rate in the presence of guanine was identical to that observed in SCDM supplemented with group B (Fig. 2).

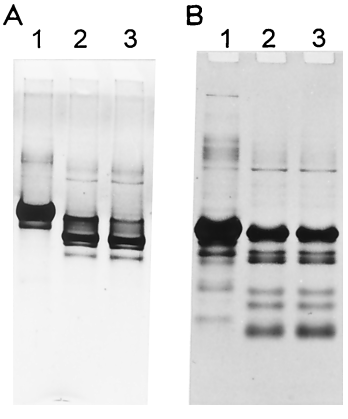

FIG. 1. SDS-PAGE analysis of α-casein (A) and β-casein (B) hydrolysis by *L. helveticus* CRL 1062 (lane 2) and S1 (lane 3) after growth in CDM. Lane 1, starting substrates.

Guanine biosynthesis in *L. helveticus* CRL 1062 and S1. To further identify the defect in S1, the activities of two key enzymes directly linked to guanine biosynthesis were determined. GMP is derived from IMP in two steps (25, 37) (Fig. 3). The first step is NAD-dependent oxidation of IMP catalyzed by IMP dehydrogenase (encoded by *guaB*), and the product formed is XMP. In the second step, the amidotransferase GMP synthetase (encoded by *guaA*) catalyzes the amidation of XMP in a glutamine- or NH_3-dependent process requiring ATP. The values of GMP synthetase were similar in both strains (12 ± 0.4 and 11 ± 0.3 μmol/min/mg of protein for CRL 1062 and S1, respectively). In contrast, IMP dehydrogenase activity in CE of S1 strain was 14 ± 0.6 μmol/min/mg of protein, which is five times lower than that exhibited by the parental strain, CRL 1062 (66 ± 3.0 μmol/min/mg of protein), suggesting that S1 was defective in this enzyme. This conclusion was further supported by the observation that the addition of guanine or xanthine to milk, a substrate poor in purine compounds, restored the Fmc^+ phenotype of *L. helveticus* S1 (Fig. 4). Thus, the pH decrease for S1 strain in RSM supplemented with xanthine or guanine was similar to that obtained for *L. helveticus* CRL 1062 with or without bases (Fig. 4). On

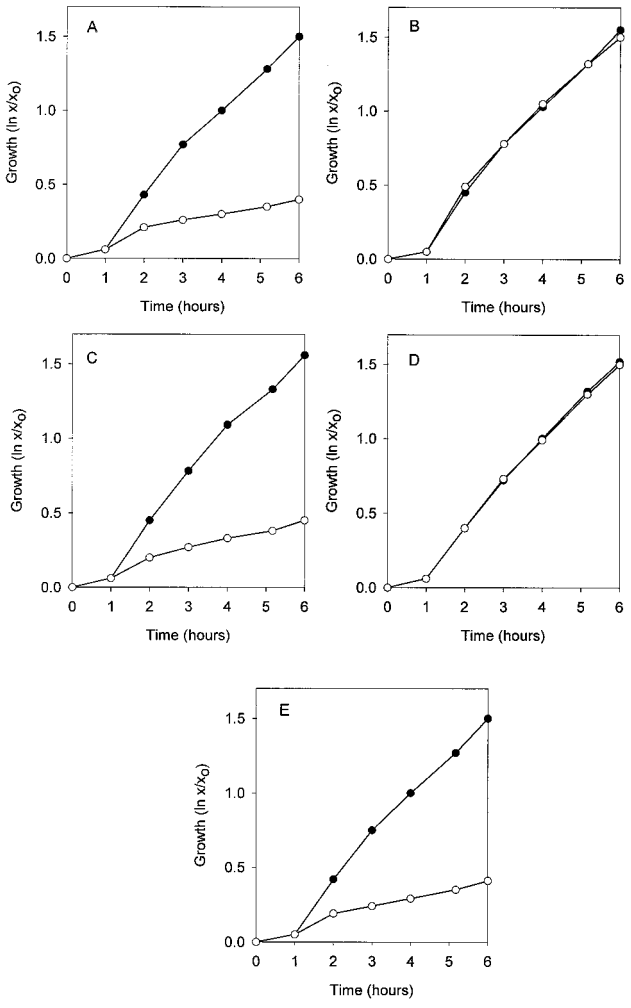

FIG. 2. Growth of *L. helveticus* CRL 1062 (●) and S1 (○) in SCDM (A), SCDM supplemented with adenine, guanine, and inosine (B), and SCDM plus adenine (C), guanine (D), or inosine (E). Cell growth was expressed as ln x/x_0, where x_0 is biomass produced at initiation of the experiment and x is biomass at the indicated time. Values are averages from three independent experiments.

the other hand, the addition of inosine or adenine to milk did not affect the coagulation rate of this strain (Fig. 4).

DISCUSSION

It has been reported that rapid growth and concomitant fast acid production of *L. helveticus* in milk, a substrate with a low content of free essential amino acids and peptides (22), depend mainly upon a proteolytic system which allows degradation of milk proteins (caseins) and on β-galactosidase activity (15). In this sense, most of the Fmc$^-$ variants of *L. helveticus* so far characterized have been putatively associated with a deficiency in proteolytic activity (2, 6, 23, 29). In the present study, however, strain S1, an Fmc$^-$ variant of *L. helveticus* CRL 1062, possessed functional β-galactosidase and proteinase activities. Furthermore, strain S1 was able to grow in leucine-free CDM supplemented with di-, tri-, or oligoleucine-containing peptides as the sole source of leucine, indicating the presence in *L.*

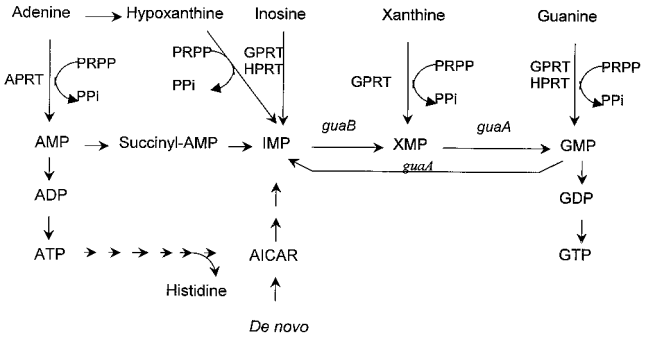

FIG. 3. Metabolic pathways potentially involved in the biosynthesis and interconversion of purines in *L. helveticus* CRL 1062. Enzymes assayed in this study are identified by their gene symbols: *guaB*, IMP dehydrogenase; *guaA*, GMP synthetase. AICAR, aminoimidazolecarboxalamide ribonucleotide; APRT, adenine phosphoribosyltransferase; GPRT, guanine phosphoribosyltransferase; HPRT, hypoxanthine phosphoribosyltransferase. Data were adapted from reference 33.

helveticus S1 of functional di-, tri-, and oligopeptide transport systems as well as functional intracellular peptidases. *L. helveticus* CRL 1062 (and hence S1) is auxotrophic for aspartate (14). Therefore, and in contrast to *L. lactis* C2 (35), the deficiency in aspartate biosynthesis in strain S1 was not responsible for its Fmc$^-$ phenotype.

Milk is also deficient in purine and pyrimidine compounds (3, 26, 31). The purine nucleotides can be formed by de novo biosynthesis, which requires in general 10 enzymatic steps leading to IMP, and by salvage reactions from purine nucleosides and bases (25, 37) (Fig. 3). A defective pathway of de novo purine biosynthesis seems to be common in most of the homofermentative lactobacilli (4). However, this study suggests that in CRL 1062, the de novo biosynthetic pathway for purine nucleotides is functional (CRL 1062 grew on SCDM) and is necessary to support fast acid production in milk and for the population to reach high cell densities. Like *L. lactis* (25), *L. helveticus* CRL 1062 was capable of converting adenine, gua-

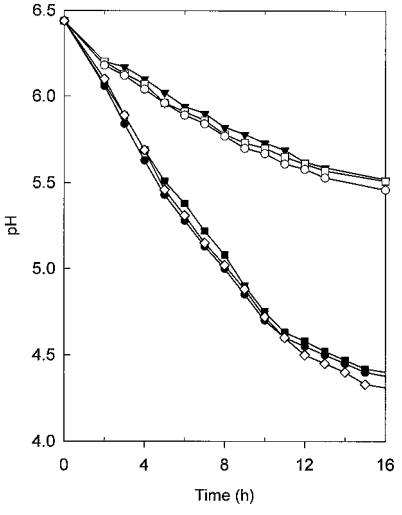

FIG. 4. Rates of acidification during cultivation of *L. helveticus* S1 in RSM without nucleotides (○), *L. helveticus* S1 in RSM supplemented with inosine (▼), adenine (□), xanthine (◇), or guanine (■), and *L. helveticus* CRL 1062 in RSM (●).

nine, and inosine to AMP, GMP, and IMP, respectively, indicating the existence of adenine phosphoribosyltransferase and hypoxanthine guanine phosphoribosyltransferase activities. It also revealed that *L. helveticus* S1 lacks the ability to convert IMP into adequate amounts of XMP, a precursor of GMP, which is due to a reduced IMP dehydrogenase activity.

Plasmid instability has explained certain intrastrain variations of *L. helveticus* (2, 23, 30). However, both CRL 1062 and S1 are plasmid free (13). Therefore, the observed phenotypic variability does not appear to be linked to variations in plasmid DNA content. It has also been reported that spontaneous and phenotypically stable mutations in the β-galactosidase locus of *L. helveticus* strains were caused by integration of ISL*2* into the gene (38). *L. helveticus* CRL 1062 contains both IS*1201* (32) and ISL*2* sequences in its chromosomal DNA (unpublished data). Comparison of Southern blot patterns hybridized with an internal fragment of ISL*2* revealed no visible differences between the S1 and wild-type strains (data not shown). These results suggest that the purine auxotrophy of S1 is due either to a point mutation or to small DNA rearrangements which were not detectable on the Southern blots. The alteration affecting the ability to synthesize guanine appears to result in the production of an altered IMP dehydrogenase with a partial loss of catalytic activity.

The simplified CM described in this work was also used to isolate Fmc$^-$ derivatives from *Lactobacillus delbrueckii* subsp. *lactis* CRL 581. Preliminary characterization of these Fmc$^-$ strains also showed that they were defective in the de novo synthesis of purine nucleotides, leading to IMP (unpublished data). We are currently investigating whether CM is suitable for isolating slowly milk-coagulating variants from other LAB.

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